NEMAGEL - A FORMULATION OF THE ENTOMOPATHOGENIC NEMATODE *STEINERNEMA THERMOPHILUM* MITIGATING THE SHELF-LIFE CONSTRAINT OF THE TROPICS

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Summary. NemaGel, a novel formulation for entomopathogenic nematodes, based on a newly developed hydrogel, has been found to enhance the shelf life of the indigenous nematode *Steinernema thermophilum*. The nematode concentration in the NemaGel can be adjusted up to 1×10^5 infective juveniles per gram. The survival of formulated nematodes was significantly better than that in aqueous suspensions after 9 months storage at 15 °C, and after 6, 2 and one weeks onward at 30, 35 and 40 °C, respectively. More than 50% survival of formulated infective juveniles was recorded even after 36 months storage at 15 °C, and 24, 16 and 8 weeks of storage at 30, 35 and 40 °C, respectively. Formulated nematodes stored at room temperature (exposed to fluctuating diurnal temperature conditions varying from 15 to 39 °C), from August, 2004 to May, 2005, showed 89% survival compared to only 16% in control, after 9 months of storage.

Key words: New formulation, Steinernematidae, survival, temperature.

Insect pests are a major constraint to maximizing the productivity of agricultural crops. The use of chemical insecticides often causes problems, such as insect resistance to chemicals, accumulation in the animal body and environmental hazards. Therefore, eco-friendly options to replace chemical pesticides are sought globally and the use of biopesticides is one of the viable approaches.

Entomopathogenic nematodes (EPNs) belonging to the families Steinernematidae and Heterorhabditidae carry symbiotic bacteria that are released in the insect hemocoel, wherein they multiply and cause septicaemia, resulting in death of the insect. In addition, the nematodes are able to recycle in the environment and are safe to human health, plants and other non-target organisms. In the USA and some other countries, these nematodes are already being used for the management of insect pests under field conditions as well as in home gardens (Gaugler and Kaya, 1990; Gaugler, 2002).

A major obstacle in the use of EPNs as bioinsecticides is their poor shelf life. This is primarily due to their sensitivity to desiccation and UV radiation and their poor tolerance of high temperature. Therefore, efforts have been made to develop formulations that will overcome these limitations (Grewal, 2002). However, no microbial formulation is known to match the 2-year shelf-life of formulations of chemical pesticides. Various carriers, such as clay (Bedding, 1991), activated charcoal (Yakawa and Pitt, 1985), sponge, vermiculite, and peat (Georgis, 1990), have been used to formulate infective juvenile nematodes. A formulation based on immobilization of nematodes in calcium alginate gel has also been developed (Georgis, 1990; Kaya and Nelsen, 1985). This formulation requires the use of sodium citrate to release the nematodes from the gel, which takes about 20-30 minutes and often limits its large scale application. To overcome this, water dispersible granules of the EPNs were developed (Georgis and Dunlop, 1994). Although convenient to use, the maximum shelf-life of the infective juveniles of EPNs achieved was six months at temperatures up to 25 °C. In some of the formulations, water soluble hydrogels have also been used (Nelsen and Catherine, 1986, 1987).

EPNs employed in most of the biopesticidal formulations are not suitable for use under tropical and subtropical conditions because of their inability to withstand temperatures above 25 °C. *Steinernema thermophilum* Ganguly *et* Singh, with a broad spectrum of biocontrol potential, has been found to be effective at wide ranges of temperature and moisture, and thus is a suitable bioagent under tropical and subtropical conditions (Ganguly and Singh, 2000, 2001; Ganguly and Gavas, 2004a, 2004b). Its field efficacy, when used as foliar spray against the diamond back moth (*Plutella xylostella*) on cabbage, was also established (Somvanshi *et al.*, 2006). To commercialise this nemic bioagent, there was an urgent need for a suitable formulation, not only to enhance its shelf-life but also to facilitate its transportation. Therefore, a novel formulation of *S. thermophilum*, namely NemaGel, was developed using super-absorbent hydrogel of semisynthetic origin (Ganguly *et al.*, 2006). This paper reports on the shelf life of *S.*

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thermophilum in NemaGel, at constant temperatures of 15, 30, 35 and 40 °C in BOD incubators, as well as varying diurnal (maximum and minimum) temperature conditions prevailing in ambient conditions.

MATERIALS AND METHODS

The novel hydrogel (Anupama *et al*., 2005) was used as a carrier to entrap and immobilize the freshly emerged infective juveniles of *S. thermophilum*, to form NemaGel. Details of the formulation are not given for patent reasons. The water level of the formulation was optimized to ensure adequate entrapment (Ganguly *et al.,* 2006). One gram of NemaGel could effectively entrap up to 1×10^5 infective EPN juveniles (Fig. 1). To study the shelf-life, three experiments were conducted. Forty grams of the formulation, containing approximately 4×10^6 infective juveniles, were placed in each of five Petri dishes of 10 cm diameter. The dishes were closed to conserve moisture and then incubated under different conditions: (i) constantly high temperatures (30, 35 and 40 °C), separately in BOD (Biological Oxygen Demand) incubators; (ii) low temperature (15 °C), in a BOD incubator, and (iii) at fluctuating diurnal temperature conditions in the laboratory at room temperature. The maximum and minimum temperatures in the laboratory were recorded every day. A control, consisting of 40 ml of an aqueous suspension containing 40,000 infective EPN juveniles placed in a tissue culture flask (150 ml capacity), was kept horizontally with each set. In the high temperature conditions, observations were made at weekly intervals up to 48 weeks, while in the low temperature and ambient conditions observations were made at monthly intervals.

An aliquot of 500 mg was drawn at weekly intervals from the Petri dishes containing NemaGel and dissolved in 50 ml water on a magnetic stirrer for 5 minutes to release the nematodes. The nematode suspensions thus obtained were observed under a stereo-microscope and checked for live and dead nematodes. The dead nematodes were confirmed as dead by prodding with a fine bamboo pick. Inactive nematodes started moving when prodded, while dead nematodes did not. The counts of live and dead nematodes/unit sample of each treatment were converted to percentages of survival of infective juveniles during storage at the different temperatures. The data thus obtained were statistically analysed and F values and critical differences were computed in order to compare treatments at different temperatures and time intervals (Tables I-III).

RESULTS AND DISCUSSION

In general, formulated nematodes had significantly better shelf-lives than the control, in all of the temperature conditions. Significant differences between survival of formulated nematodes and those in aqueous suspensions were noticed after 6 weeks at 30 °C, after 2 weeks at 35 °C, one week at 40 °C, and after 9 months at 15 °C, as well as at fluctuating room temperature (Tables I-III).

Table I. Per cent survival of infective juveniles of *Steinernema thermophilum* during storage at different temperatures in BOD incubators up to 48 weeks.

Temp. (°C)	Treatment	Per cent survival after weeks								
		1	2	4	6	8	16	24	36	48
30	Formulated	99	97	97	96	91	86	50	25	0
	Control	97	95	90	78	60	32	16	7	0
35	Formulated	96	94	89	82	71	50	33	12	0
	Control	94	85	65	35	20	9	0	-	-
40	Formulated	88	83	79	73	54	14	0	-	-
	Control	74	67	42	0	-	-	-	-	-

Statistical Analysis

Source	Degree of freedom	Sum of squares	Mean square	F value	Pr > F	Critical difference
Treatment (weeks)	7	42617.15	6088.16	34.05	<.001	15.62
Temperature	2	9182.79	4591.39	25.68	<.001	9.58
Formulation	1	7375.52	7375.52	41.25	<.001	7.82
Error	37					
R^2			0.899			
Root MSE			13.37			

Table II. Per cent survival of infective juveniles of *Steinernema thermophilum* during storage at 15 °C in a BOD incubator up to 36 months.

Treatment	Per cent survival after months								
	1	2	4	6	9	12	18	24	36
Formulated	100	100	100	100	100	96	88	73	58
Control	100	100	100	99	85	73	67	38	14

Statistical Analysis

Source	Degree of freedom	Sum of squares	Mean square	F value	Pr > F	Critical difference
Treatment (months)	8	8454.0	1056.75	7.65	0.0047	27.10
Formulation	1	1073.39	1073.39	7.77	0.0236	12.77
Error	8	1105.11	138.14			
R2			0.8960			
Root MSE			11.75			

The infective juveniles (IJs) of *S. thermophilum* entrapped in the hydrogel matrix exhibited 86% survival at 30 °C after 16 weeks of storage, and 50% after 24 weeks of storage. Formulated nematodes had shelf-lives of 16 and 8 weeks at 35 and 40 °C, respectively, with 50% of the nematodes still alive and active. In comparison, the control treatments showed less than 50% survival after 16, 6 and 4 weeks at 30, 35 and 40 °C, respectively (Table I). The survival of IJs in NemaGel was significantly better than that of nematodes in aqueous suspensions after 6, 2 and one week onwards, respectively, at 30, 35 and 40 °C. The results demonstrated an improved shelf-life of the bioagent in this formulation, even at high temperatures.

Storage of the test formulation at 15 °C resulted in 96% survival of the juveniles after 12 months and 58% after 36 months (Table II). In the control, the survival declined after 9 months and was reduced to only 14% after 36 months. Up to 6 months, the survival of IJs in the formulation was 100%, and the control (99%) was almost at par. But, at 9 months onwards, formulated nematodes showed significantly better survival than the control. This feature has not been reported earlier in the literature, thus underlining the potential of the new formulation.

The shelf life of the new formulation was additionally evaluated under ambient conditions in the laboratory from August 2004 to May 2005, thus exposing it to diurnal as well as seasonal variations in temperature. The temperature during this period varied from 15 to 39 °C (Table III). Survival was sustained until November 2004, with temperatures varying from 18 to 30 °C. In February 2005, 98% viable juveniles were recorded with the temperature varying from 15 to 23 °C during this period. In May 2005, when there was sudden rise in temperature (28-39 °C) at the onset of summer, the formulation exhibited 89% survival, compared to only 16% in the control. This observation sets this formulation apart from other formulations that have been tried so far.

The main features of NemaGel and the two best known previous formulations, viz., water dispersible granules and alginate gel, developed for exotic strains of steinernematids, are presented in Table IV.

An important factor in temperature tolerance by nematodes and their adaptation to extreme environmental conditions is lipid saturation (Selvan *et al.*, 1993). Most of the bio-formulations of EPNs developed so far report significant shelf lives only up to 25 °C and for a maximum of 12 weeks. *Steinernema carpocapsae* (Weiser,

Table III. Per cent survival of infective juveniles of *Steinernema thermophilum* during storage from August, 2004 to May, 2005, under laboratory fluctuating conditions and different temperature ranges.

Treatment	Per cent survival during August 2004 – May 2005				
	Aug. 2004 (27-35 °C)	Sept. 2004 (25-30 °C)	Nov. 2004 (18-25 °C)	Feb. 2005 (15-23 °C)	May 2005 (28-39 °C)
Formulated	100	100	100	98	89
Control	100	98	97	96	16

Statistical analysis

Source	Degree of freedom	Sum of squares	Mean square	F value	Pr > F	Critical difference
Treatment	4	3413.4	853.3	1.68	0.314	62.59
Formulation	1	640.0	640.0	1.26	0.3246	39.56
Error	4	2033.0	508.2			
R^2			0.666			
Root MSE			22.54			

Table IV. A comparison of two previously known formulations with the newly developed NemaGel.

Characteristics	NemaGel	Water Dispersible Granule	Alginate gel*
Bioagent (*Steinernema* species used)	*S. thermophilum*	*S. carpocapsae*	*S. carpocapsae*
Product description	Nematodes entrapped and immobilized in water insoluble hydrogel matrix	Nematodes encased in granules	Nematodes immobilized in calcium alginate gel and coated on a mesh screen
Nematode status	Immobilized	Partially desiccated	Immobilized
Shelf-life[a]	18-24 months at 15 °C; 16 weeks at 30 °C; 6-8 weeks at 35 °C; 2- 4 weeks at 40 °C	6 months at 4-25 °C; 2 months at 30 °C; 6 days at 36 °C	5 months at 4-25 °C; 2 weeks at 30 °C; A few hours at 36 °C
Ease of use	Ready to use gel; can be applied directly at the time of sowing, based on crop specific requirement	Dissolves immediately in water, easy to measure	20-30 minutes preparation time required; once diluted, the entire product must be used
Product size: coverage ratio	Adequate for use in diverse agricultural use situations	Adequate for use in various markets	Adequate only in green house and kitchen gardens
Use of chemical agents at the time of application	Not required	Not required	Sodium citrate required
Cost	Lower than both WDG and alginate	Low for ingredients and manufacture	Higher than WDG

* From Georgis and Dunlop, 1994; [a] More than 80% survival.

1955) Wouts *et al*., 1982, immobilised in 20% calcium alginate, had a maximum shelf life of 3 months at 25 °C for a 90% survival rate (Kaya and Gaugler, 1993). Another steinernematid, *S. glaseri* (Steiner, 1929) Wouts *et al.,* 1982, also had a shelf life of 12 weeks at 25 °C, when formulated in 35% calcium alginate beads. Beyond 35 °C, the juveniles could not survive for more than one month (Georgis and Kaya, 1998). Another formulation of *S. carpocapsae*, as water-dispersible granules (WG), easier to use than calcium alginate, had a maximum shelf-life of 5-6 months at 4-25 °C, 2 months at 30 °C and 6 days only at 36 °C (Georgis and Dunlop, 1994; Grewal, 2002), which is much less than the survival in NemaGel in the present study.

Compared to other known formulations, the longer shelf-life of the NemaGel, especially at high temperatures, might be attributed partly to the inherent heat tolerant capability of *S. thermophilum*, the bioagent used (Ganguly and Singh, 2001). The optimum temperature for most of the exotic strains is about 22 °C, which is much less than that of *S. thermophilum.* Enhanced shelf-life of the nematodes in NemaGel could also be attributed to certain intrinsic properties of the carrier gel used in the formulation, because formulated nematodes exhibited much better shelf-life compared to those of aqueous suspensions. When compared with the existing formulations, namely calcium alginate gel and water dispersible granules, the new formulation revealed certain advantageous features (Table IV). The test hydrogel matrix of the new formulation in which the nematodes are entrapped is transparent, thus permitting a clear view of the nematodes when counting them (Fig. 1). Since there is no extra coating material, this formulation can hold up to 100,000 nematodes per gram. Apart from entrapping nematodes, the matrix is reported to hold large amounts of water and nutrients (Anupama *et al.*, 2005), thus serving as a water and nutrient management aid for the plants grown in the treated soil.

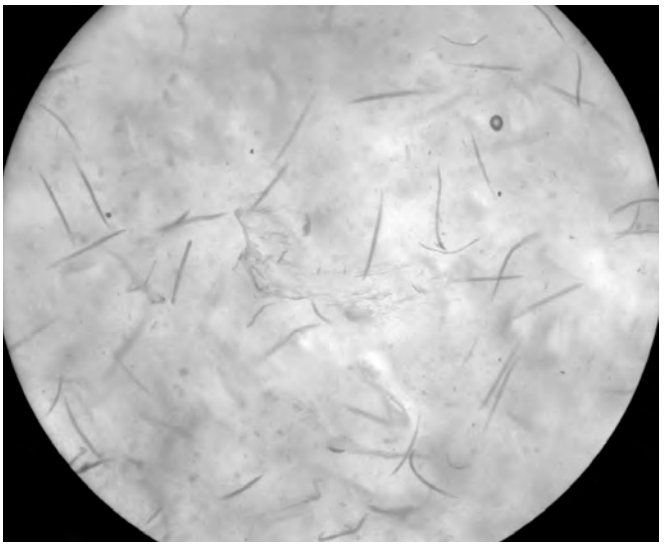

Fig. 1. Microphotograph showing infective juveniles of *S. thermophilum* entrapped in the transparent matrix of NemaGel.

Thus, it can be concluded that the newly developed nematode based biopesticidal formulation in hydrogel, employing entrapment and immobilization of heat tolerant *S. thermophilum*, a steinernematid of Indian origin, possesses advantageous features that can be exploited in ecofriendly insect pest, water and nutrient management.

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